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Molecular analysis of the interaction between cardosin A and phospholipase Dα

Identification of RGD/KGE sequences as binding motifs for C2 domains

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Cardosin A is an RGD-containing aspartic proteinase from the stigmatic papillae of *Cynara cardunculus* L. A putative cardosin A-binding protein has previously been isolated from pollen suggesting its potential involvement in pollen–pistil interaction [Faro C, Ramalho-Santos M, Vieira M, Mendes A, Simões I, Andrade R, Verissimo P, Lin X, Tang J & Pires E (1999) *J Biol Chem* **274**, 28724–28729]. Here we report the identification of phospholipase Dα as a cardosin A-binding protein. The interaction was confirmed by coimmunoprecipitation studies and pull-down assays. To investigate the structural and molecular determinants involved in the interaction, pull-down assays with cardosin A and various glutathione *S*-transferase-fused phospholipase Dα constructs were performed. Results revealed that the C2 domain of phospholipase Dα contains the cardosin A-binding activity. Further assays with mutated recombinant forms of cardosin A showed that the RGD motif as well as the unprecedented KGE motif, which is structurally and charge-wise very similar to RGD, are indispensable for the interaction. Taken together our results indicate that the C2 domain of plant phospholipase Dα can act as a cardosin A-binding domain and suggest that plant C2 domains may have an additional role as RGD/KGE-recognition domains.

Aspartic proteinases are widely distributed among plant species [1]. Like most other members of this protease family, they are mainly active at acidic pH, are specifically inhibited by pepstatin and have two aspartic acid residues that are indispensable for catalytic activity [2,3]. Determination of the 3D structure of two plant aspartic proteinases has also shown that they share significant structural similarity with other known structures of aspartic proteinases from different eukaryotic sources [4,5]. Cardosin A is one of the plant aspartic proteinases that has had its structure determined [4]. Together with cardosin B, they constitute model plant aspartic proteinases comprising the structural features that characterize the majority of plant aspartic proteinases identified so far [1].

Cardosins A and B are highly expressed in the pistils of the cardoon *Cynara cardunculus* L, the milk-clotting activity of which has been used in traditional cheese making processes [6]. They are both synthesized as single-chain preproenzymes comprising a signal peptide,

Abbreviations
GST, glutathione *S*-transferase; pCA, procardosin A; PLD, phospholipase D; RACE, rapid amplification of cDNA ends.

a prosegment and a saposin-like domain (plant-specific insert sequence), which are all removed to yield mature and glycosylated two-chain enzymes [4,7,8]. Although both cardosins cleave peptide bonds between bulky hydrophobic amino acids, cardosin B displays a broader substrate specificity and higher proteolytic activity than cardosin A [9]. Different histological and cytological localizations have also been reported for these enzymes. Whereas cardosin A is predominantly accumulated in protein storage vacuoles and also found at the cell wall of stigmatic papillae, cardosin B is an extracellular protein present in the transmitting tissue of the pistil. The differences in activity and localization have suggested that they may fulfil different biological functions, with cardosin B taking part in general protein degradation whereas cardosin A may play a role in a more specifically regulated process [8,10].

In a previous paper, a protein that specifically interacts with cardosin A was isolated from pollen extracts of cardoon [7]. Elution of this protein from a cardosin A–Sepharose column after addition of an RGD-containing peptide suggested that cardosin A, which contains a unique RGD motif (residues 246–248 of the full-length cDNA-derived amino-acid sequence) in its sequence, may be involved in protein–protein interaction through an RGD-dependent recognition mechanism. In mammalian cells, the fundamental role of the RGD-mediated interaction between integrins and their ligands for the activation of essential signalling pathways in cell proliferation and growth has been well studied [11]. In contrast, the identification of functional homologues of integrins or adhesion proteins in plants and their biological relevance remains to be established. Thus far, there are several reports showing the effect of RGD peptides on different plant processes and immunological evidence of the presence of integrin-like and adhesion molecule homologues [12–27]. However, an RGD-containing protein and its interacting partner have not been identified in plants.

In this work, we report the identification of phospholipase D (PLD)α as the cardosin A-binding protein and describe the involvement of the RGD motif as well as the charge-wise similar KGE sequence (residues 455–457) in the interaction between these two plant proteins.

Results

Purification and identification of cardosin A-interacting protein

We have previously described the purification of a cardosin A-binding protein from pollen extracts after elution with an RGD-containing peptide [7]. This result indicated that the RGD motif present at the surface of cardosin A may be involved in the interaction between these two proteins. To identify the cardosin A-interacting protein from the pollen of *Cynara cardunculus* L, the protein was purified by affinity chromatography on a NHFRGDHTK–Sepharose column (synthetic peptide designed from the amino-acid sequence of cardosin A). Two proteins with apparent molecular masses ≈90 kDa and 67 kDa were isolated on elution with an RGDS peptide (Fig. 1A). The 90-kDa protein has a molecular mass similar to that of the protein isolated by cardosin A–Sepharose affinity chromatography [7], whereas the 67-kDa protein was eluted only on the NHFRGDHTK–Sepharose affinity chromatography. MS analysis of the 90-kDa protein allowed us to obtain several partial amino-acid sequences (Table 1). These peptide sequences showed very high similarity to various PLDα enzymes from different plant species, providing the first strong clue to the identity of the cardosin A-interacting protein. This initial assumption was further strengthened by

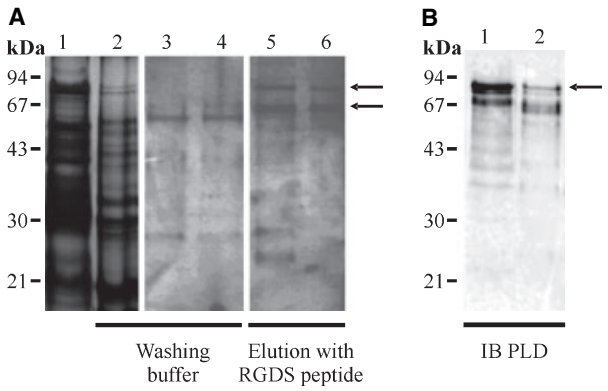

Fig. 1. (A) Purification of a cardosin A-interacting protein by NHFRGDHTK–Sepharose 4B affinity chromatography. An octyl glucoside pollen extract was applied to a NHFRGDHTK–Sepharose 4B column. The amino-acid sequence of the synthetic peptide used as ligand is the same as found in cardosin A around the RGD motif. Elution was achieved with buffer containing the commercial peptide RGDS (1 mg·mL^{-1}). Collected fractions were analyzed by SDS/PAGE in 12% polyacrylamide gels and visualized by silver staining. Lane 1, octyl glucoside pollen extract; lanes 2–4, washing fractions; lanes 5 and 6, fractions eluted with RGDS peptide (1 mg·mL^{-1}). The arrows indicate the two proteins of 90 kDa and 67 kDa copurified in this chromatography. (B) PLDα is purified either by NHFRGDHTK–Sepharose or cardosin A–Sepharose affinity chromatography. Elution fractions from NHFRGDHTK–Sepharose 4B (lane 1) and cardosin A–Sepharose (lane 2) affinity chromatography were analyzed by immunoblotting with an antibody raised against cabbage PLDα (IB PLD). The arrow indicates the 90-kDa protein cross-reaction with the PLDα antibody.

Table 1. MS-sequenced and identified peptides of PLDα from *C. cardunculus* L. Database searches with the partial amino-acid sequences revealed high sequence similarity with the PLDα sequence from *N. tabacum* (accession number P93400).

Mass (Da)	Theoretical mass (Da)	Position	Peptide sequence
1240.68	1240.64	001–012	DDNPIGATLIGR
1131.54	1131.52	019–028	ELLDGDEVDK[a]
1444.74	1444.71	070–081	YPGVPYTFFAQR
1841.98	1841.93	086–101	VSLYQDAHVPDNFIPK[a]
1103.56	1103.54	172–180	VALMVWDDR
1371.69	1371.65	216–228	DPDDGGSILQDLK
1175.62	1175.66	239–248	IVVVDHELPR
3558.68	3558.63	270–301	YDSAFHPLFSTLDSAHHDDFHQPNYAGASIAK[a]
1175.62	1175.55	306–314	EPWHDIHSR
1894.88	1894.86	372–390	SIDGGAAFGFPDTPEEASK[a]
1262.68	1262.66	404–414	SIQDAYINAIR
1834.98	1834.93	436–452	SDDIDVDEVGALHLIPK[a]
1015.52	1015.52	504–512	DIVDALQDK[a]
2575.14	2575.11	535–557	SGEYEPTEAPEPDSGYLHAQENR[a]
2319.10	2319.10	592–612	DSEIAMGAYQPYHLATQTPAR[a]

[a] These sequences were confirmed with the protein sequence deduced from the DNA sequence.

Western blotting analysis using an antibody raised against cabbage PLDα that cross-reacted with our 90-kDa cardosin A-binding protein (Fig. 1B). After the identification of cardoon PLDα as a cardosin A-binding protein, we examined whether cardosin A is associated with PLDα *in vivo*. Immunoprecipitation using a purified polyclonal antibody against cabbage PLDα resulted in the specific coimmunoprecipitation of cardosin A in both male and female reproductive organs (Fig. 2A). The specificity of the signal detected for cardosin A was confirmed by blocking the immunodetection of this protein after preincubation of the antibody against recombinant cardosin A with native cardosin A (Fig. 2B).

Molecular cloning of *C. cardunculus* L PLDα cDNA and characterization of the deduced amino-acid sequence

To characterize further cardoon PLDα, which was identified as the cardosin A-binding protein, we cloned its cDNA. In the first step, different combinations of degenerate primers encoding amino-acid sequences determined by MS/MS (Table 1) were used to PCR-amplify internal fragments of the cDNA. The nature of the fragments was confirmed by DNA sequencing and by comparison with the known partial amino-acid sequences. Specific internal primers were then designed based on the sequence of these cDNA fragments, and

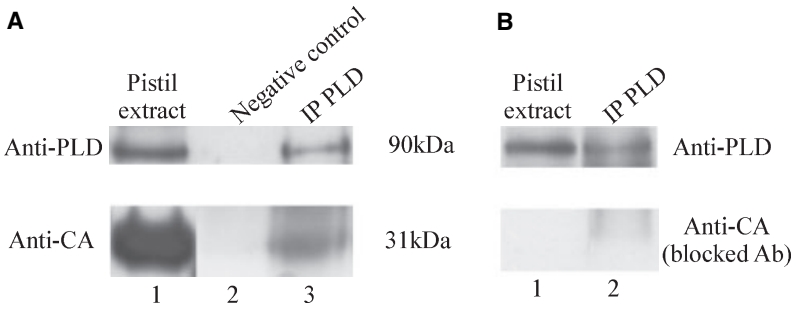

Fig. 2. Cardosin A associates with PLDα *in vivo*. (A) PLDα was immunoprecipitated from pistil extracts of *C. cardunculus* L with a purified polyclonal antibody against cabbage PLDα. The immunoprecipitate was analyzed by western blotting using PLD antibody (upper panel) and a monospecific recombinant cardosin A antibody (lower panel). Lane 1, whole extracts of mature pistils used as a positive control; lane 2, pistil extracts incubated with protein A–Sepharose in the absence of PLD antibody (negative control); lane 3, immunoprecipitation with the PLD antibody (IP PLD). (B) Cardosin A antigen control (cardosin A antibody preincubated with purified native cardosin A). Lane 1, whole extracts of mature pistils; lane 2, immunoprecipitation with the PLD antibody (IP PLD). Immunodetection was performed with PLD antibody (upper panel) and blocked recombinant cardosin A antibody (lower panel).

the 5′ and 3′ regions of PLDα cDNA were amplified by rapid amplification of cDNA ends (RACE). The complete 808-amino-acid sequence, deduced from the 2424-bp cDNA fragment, and the alignment with amino-acid sequences from *Arabidopsis thaliana* and *Nicotiana tabacum* PLDα (accession numbers Q38882 and P93400, respectively) are shown in Fig. 3. Cardoon PLDα displayed 74% sequence identity with *Arabidopsis* PLDα and 77% with tobacco PLDα. The HKD motif, crucial for catalytic activity of PLD and repeated twice in all cloned enzymes [28], was identified in the sequence. Furthermore, it was possible to confirm the presence of the 'IYIENQFF' motif, a highly conserved domain almost as critical as the HKD motif for activity and only found in PLD family members that exhibit *bona fide* PLD activity [29]. The C2 domain, a well-described regulatory Ca^{2+}/phospholipid-binding domain [30], is also present at the N-terminus of cardoon PLDα, and three highly conserved known Ca^{2+}-coordinating amino acids (Asn69,

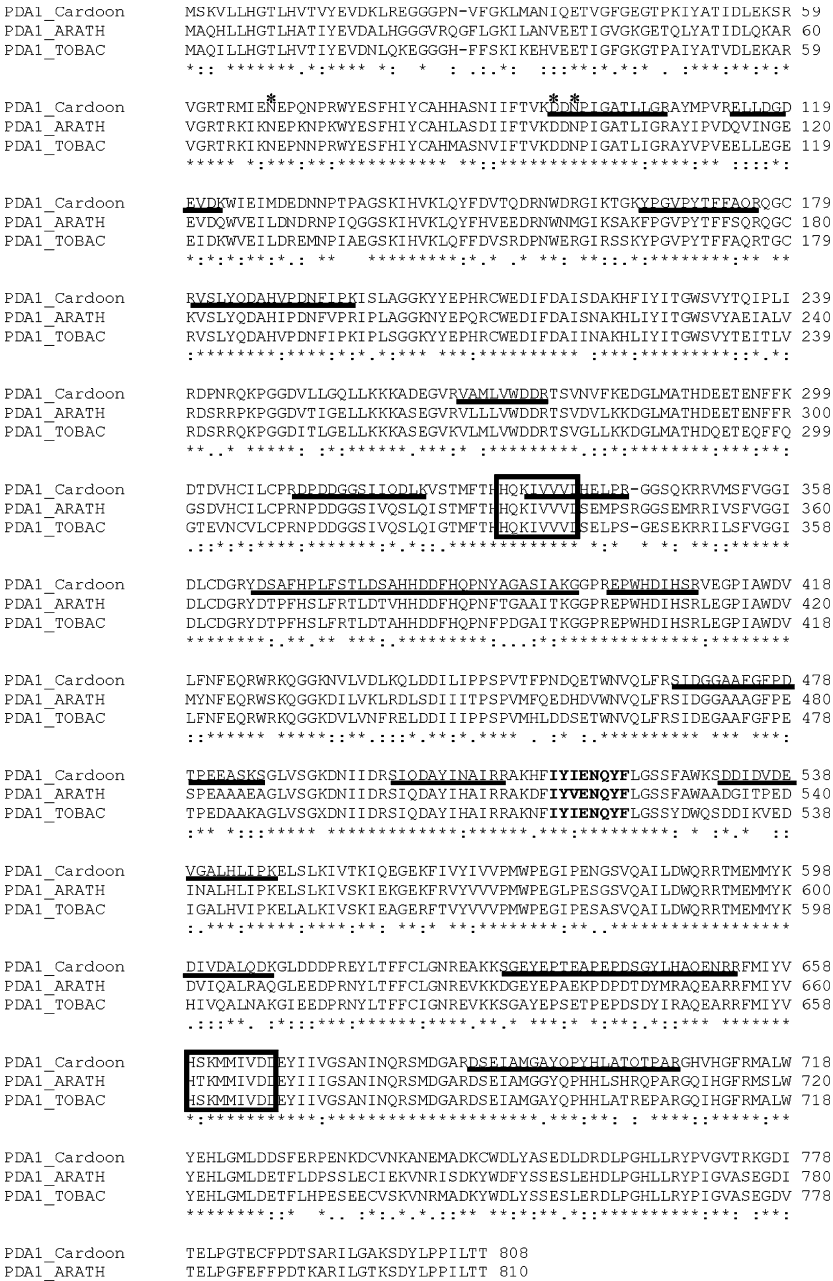

Fig. 3. Deduced amino-acid sequence of PLDα from *C. cardunculus* L and protein sequence alignment with PLDα from *A. thaliana and N. tabacum*. The complete 808-amino-acid sequence deduced from the 2424-bp cDNA of cardoon PLDα (PDA1_cardoon; accession number AJ583515) displayed 74% sequence identity with *A. thaliana* PLDα (PDA1_ARATH) and 77% with tobacco PLDα (PDA1_TOBAC; accession numbers Q38882 and P93400, respectively). The two HKD catalytic motifs are boxed and the 'IYIENQFF' motif is in bold. The first 150 amino acids of cardoon PLDα correspond to the C2 domain. Three highly conserved Ca^{2+}-coordinating amino-acid residues are marked with an asterisk. The sequences underlined correspond to the partial amino acid sequences obtained by MS/MS (Table 1).

Asp97, Asn99; *A. thaliana* numbering) are highlighted in the alignment.

The C2 domain is sufficient to promote binding of PLDα to cardosin A

To identify the structural elements involved in recognition of cardosin A, PLDα was expressed as a fusion protein with glutathione *S*-transferase (GST-PLDα) and used in pull-down assays with native cardosin A purified from pistils of *C. cardunculus* L. Cardosin A binds specifically and directly to PLDα fused to GST, and no binding was observed when GST alone was used as a negative control (Fig. 4A, compare lanes 2 and 4) or when native cardosin B was tested in the binding assays with PLDα (Fig. 4B), confirming the specificity of the interaction between PLDα and cardosin A.

A characteristic feature of plant PLDα is the C2 domain at the N-terminus [28,31], which has previously been assumed to mediate protein–protein interactions in addition to its well-known membrane-targeting function [30]. To test whether cardosin A was interacting with the C2 domain, this N-terminal PLDα domain was fused to GST (GST-C2), expressed in *Escherichia coli* and used in pull-down assays. In these experiments, cardosin A binds consistently to the C2 domain (Fig. 5, lane 2), indicating therefore that this domain of PLDα is required and sufficient to promote the interaction between the two proteins. Cardosin A inhibition by pepstatin A resulted in no complex formation, suggesting that small conformational changes may affect this interaction (Fig. 5, lane 3). To test further the specificity of the interaction, native cardosin B was used in the binding assays. Despite the high similarity between the two pistil aspartic proteinases, neither the RGD nor the similar KGE sequence motifs are conserved in cardosin B (cardosin B contains RGN and EGE, respectively). As expected, cardosin B was unable to bind to the C2 domain, thereby confirming the selectivity of PLDα for cardosin A (Fig. 5, lane 4). Pull-down assays with GST-C2 and cardosin A performed in the presence of 0.2 mM Ca^{2+} with and without 2 mM EGTA, respectively, gave identical results and therefore suggest that this interaction is calcium independent.

Interaction between cardosin A and PLDα is mediated through RGD and KGE sequences

The RGD motif of cardosin A is located at the surface of the protein [4], as seen in other structures of biologically active proteins [32,33]. However, a careful examination of the X-ray structure of cardosin A (PDB code 1B5F) revealed also a KGE motif at the tip of a loop protruding away from the core of the protein. This amino-acid motif mimics RGD in terms of charge and is positioned at the tip of a loop and is therefore reminiscent of RGD sequences present in integrin-binding molecules because of its exposed location. On the basis of these structural findings, it was hypothesized that the interaction between PLDα and cardosin A may be mediated by either RGD or KGE sequence motifs. To test which motif was responsible

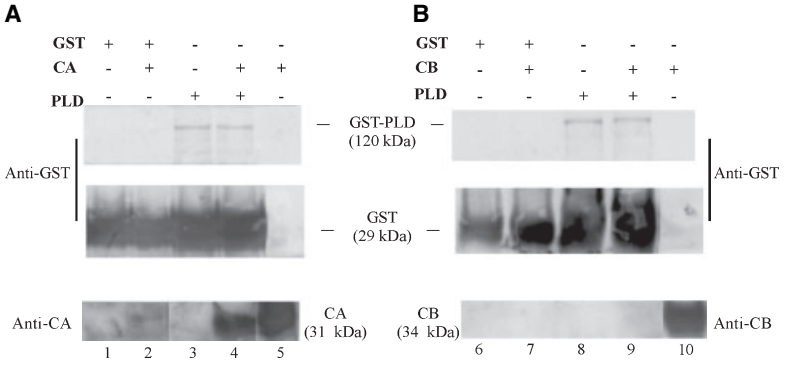

Fig. 4. Cardosin A associates directly with PLDα. (A) Binding assays for cardosin A were performed with GST alone or with GST-PLDα fusion protein. Pull-down samples were analyzed by western blotting using a GST antibody that recognizes both GST-PLDα fusion protein (upper panel) and GST (middle panel), and an antibody against recombinant cardosin A (lower panel). Lane 1, GST without cardosin A; lane 2, cardosin A incubated with GST (negative control); lane 3, GST-PLDα without cardosin A; lane 4, cardosin A incubated with GST-PLDα; lane 5, cardosin A alone. (B) Binding assays for cardosin B were performed as described for cardosin A. Pull-down samples were analyzed by western blotting using a GST antibody that recognizes both GST-PLDα fusion protein (upper panel) and GST (middle panel), and an antibody against recombinant cardosin B (lower panel). Lane 6, GST without cardosin B; lane 7, cardosin B incubated with GST (negative control); lane 8, GST-PLDα without cardosin B; lane 9, cardosin B incubated with GST-PLDα; lane 10, cardosin B alone.

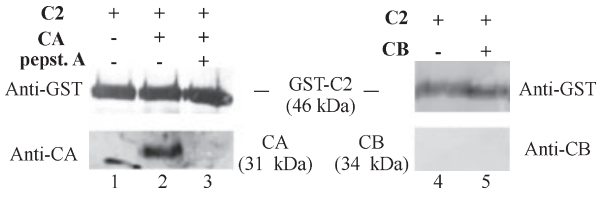

Fig. 5. Cardosin A interacts with the C2 domain of PLDα. Pull-down assays for cardosins A and B were performed with GST-C2 domain fusion protein. Pull-down samples were analyzed by western blotting using an anti-GST Ig (upper panel) and antibodies against recombinant cardosin A and cardosin B (lower panels). Lane 1, GST-C2 domain without cardosin A; lane 2, cardosin A incubated with GST-C2 fusion protein; lane 3, cardosin A incubated with GST-C2 fusion protein in the presence of pepstatin A; lane 4, GST-C2 domain without cardosin B; lane 5, cardosin B incubated with GST-C2 fusion protein.

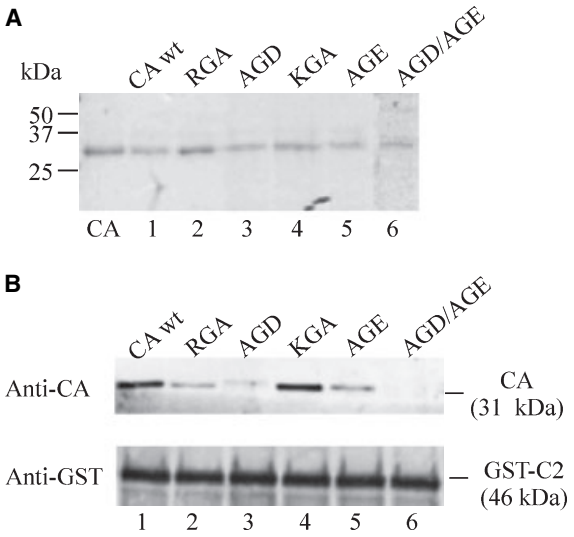

Fig. 6. Interaction between cardosin A and the C2 domain of PLDα is mediated through the RGD/KGE sequence motifs. (A) Recombinant wild-type cardosin A (lane 1) and several mutants where the RGD and KGE sequences were substituted for RGA (D248A) (lane 2), AGD (R246A) (lane 3), KGA (E457A) (lane 4), AGE (K455A) (lane 5), and AGD/AGE (R246A/K455A) (lane 6) were expressed in *E. coli* and autoactivated at acidic pH [34]. Activated samples were analyzed by SDS/PAGE, and native cardosin A (CA) was used as control. The gel was stained with Coomassie Blue. (B) After activation, recombinant wild-type cardosin A and the different mutants were used in binding assays with the GST-C2 fusion protein. Pull-down samples were analyzed by western blotting using an antibody against recombinant cardosin A (upper panel) and a GST antibody that recognizes GST-C2 fusion protein (lower panel). Lane 1, recombinant wild type cardosin A (CAwt) (positive control); lane 2, CA mutant RGA (D248A); lane 3, CA mutant AGD (R246A); lane 4, CA mutant KGA (E457A); lane 5, CA mutant AGE (K455A); lane 6, CA double mutant AGD/AGE (R246A/K455A).

for the determined interaction, several single mutants of procardosin A (pCA) were generated in which the RGD and KGE sequences were substituted for AGD (R246A), RGA (D248A), AGE (K455A) and KGA (E457A). Together with recombinant wild-type cardosin A, these mutants were expressed in *E. coli* and purified. They were autoactivated at acidic pH as previously described [34], and full aspartic proteinase activity was measured for all enzymes. The activated fractions are shown in Fig. 6A. Pull-down assays with these enzymatically active proteins and the C2 domain fused to GST revealed that both sequence motifs participate in the interaction, However, the predominant role can be attributed to the RGD sequence (Fig. 6B). Moreover, the results allow the identification of the positive residues of both motifs as the main contributors to the interaction. As shown in Fig. 6B, both RGD mutants showed a lower capacity to bind to the C2 domain when compared with wild-type recombinant cardosin A (compare lane 1 with lanes 2/3). However, whereas the AGD mutant had lost C2-binding capability almost completely, the second RGA mutant, containing the positively charged residue, had retained C2-binding capacity. Similar findings were obtained for the two KGE mutants, with the KGA mutant behaving like wild-type recombinant cardosin A whereas the substitution of the lysine residue (AGE) resulted in significantly decreased binding to the C2 domain (compare lane 1 with lanes 4/5). To confirm further the role of the two basic residues in the interaction, the double mutant AGD/AGE (R246A/K455A) was also generated (Fig. 6A, lane 6). As expected, no binding at all was observed when this mutant was used in binding assays with the C2 domain (Fig. 6B, lane 6). As previously shown for native cardosin A, no complex formation was observed when GST alone was used as a negative control. These results indicate that the basic residues in RGD/KGE motifs play an important role in the recognition of the C2 domain.

The C2 domain is degraded by cardosin A after complex disruption

After establishing the importance of RGD-like sequences in cardosin A–C2 domain complex formation and in order to examine how complex formation/disruption may affect each interacting partner, we performed pull-down assays in the presence of an RGD-containing peptide between native cardosin A and the C2 domain fused to GST. As shown in Fig. 7, the cardosin A–C2domain complex was disrupted (lane 3) or its formation impaired (lane 5) when the peptide was present in the binding assays, and this complex disruption resulted in C2 domain cleavage by

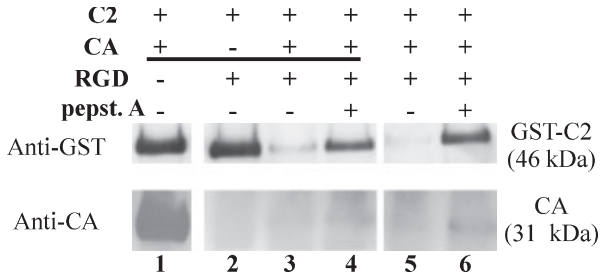

Fig. 7. C2 domain is degraded by cardosin A after complex disruption. Binding assays for cardosin A were performed with GST-C2 fusion protein in the presence of a 1.15 mM RGD-containing peptide. Pull-down samples were analyzed by western blotting using a GST antibody that recognizes GST-C2 fusion protein (upper panel) and an antibody against recombinant cardosin A (lower panel). Lane 1, cardosin A incubated with GST-C2 fusion protein (positive control); lane 2, GST-C2 incubated with the peptide NHFRGDHT; lane 3, after overnight incubation of cardosin A with GST-C2, the synthetic peptide NHFRGDHT was added and incubated for another 5 h; lane 4, same as lane 3 but incubation with the peptide was performed in the presence of pepstatin A; lanes 5–6, overnight incubation of cardosin A, GST-C2 and the peptide NHFRGDHT in the absence (lane 5) or presence (lane 6) of pepstatin A.

cardosin A. To test further the specificity of C2 degradation by cardosin A, we also performed incubation with the RGD-containing peptide in the presence of pepstatin A where no degradation of the C2 domain was observed (Fig. 7, lanes 4 and 6). Together, these results suggest that the C2 domain is a target for cardosin A and that complex formation may be a way to protect the C2 domain from cleavage.

Discussion

Cardosin A is unique among known plant aspartic proteinases in having an RGD motif located at the surface of the protein [4]. The presence of this well-known integrin-binding motif [11], and the previous purification of a cardosin A-binding protein from pollen, raised the idea that this aspartic proteinase may be involved in a adhesion-dependent recognition mechanism [7]. We have now identified the high-molecular-mass cardosin A-binding protein as PLDα. The protein was purified by affinity chromatography, and the partial amino-acid sequences obtained by MS/MS provided strong hints about its identity. Furthermore, analysis of the fractions eluted in either cardosin A–Sepharose or immobilized NHFRGDHTK affinity chromatography by immunoblotting clearly showed that, in both cases, the purified high-molecular-mass protein cross-reacts with the PLD antibody. The specificity of the interaction between cardosin A and PLDα was further confirmed in coimmunoprecipitation studies. Thus, the evidence presented here strongly indicates that PLDα is a cardosin A-binding protein.

Plant PLDαs are involved in many cellular processes, and, besides their role in membrane degradation/lipid turnover during senescence or stress responses [28,35–40], roles in signalling cascades are also emerging for this type of enzyme [28,41–46]. Both plant PLDα and aspartic proteinases have been implicated in cellular responses to biotic and abiotic stress injuries [1,28,47]. The complex formation determined between cardosin A and PLDα suggests possible concerted and/or synergistic actions in degenerative processes such as those observed during stress responses, plant senescence and/or pollen–pistil interactions. As recently shown for vacuolar processing enzyme [48], a cysteine protease implicated in vacuole-mediated cell death during hypersensitive responses, cardosin A, which is also an abundant vacuolar protease [10], may well be an important participant in vacuolar collapse-triggered cell death. Its association with PLDα may facilitate disintegration of the vacuoles in the dismantling phase of a vacuolar-type cell death. However, how this is accomplished *in vivo* remains to be elucidated.

Evaluation of structural determinants involved in the interaction between cardosin A and PLDα showed that the RGD motif in cardosin A plays an essential role in complex formation. However, we also showed that an additional KGE sequence in cardosin A also has a role in this interaction. In fact, this KGE sequence, which is located at the tip of a rather long loop, is remarkably similar in terms of charge distribution and location to RGD motifs found in biologically important proteins [32,33]. This finding is illustrated by the superimposition of the 3D structures of kistrin [32] and cardosin A (Fig. 8). The importance of both motifs and in particular their basic residues was further emphasized by the complete lack of interaction between the C2 domain and the double mutated (AGD/AGE) cardosin A. The docking model shown in Fig. 9 further highlights the role of RGD and KGE in complex formation. Moreover, it appears that the global structure of cardosin A is critical for this interaction. In fact, pepstatin-inhibited cardosin A was not able to bind to the C2 domain (Fig. 5, lane 3), indicating that conformational changes in the aspartic proteinase can prevent complex formation.

Despite some evidence of a functional role for RGD in plant development, mechanoperception and interaction with micro-organisms [12,14,15,19,20,22], there are no reports on the true nature of the RGD-containing proteins and their interacting partners. The involvement of the PLDα C2 domain in these

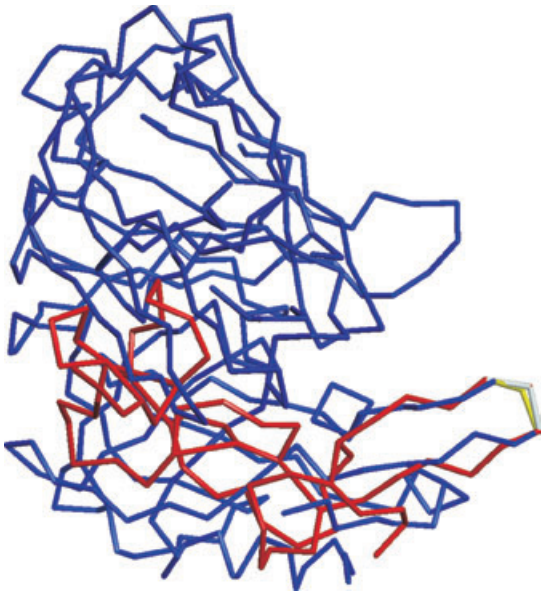

Fig. 8. The 3D structures of kistrin (PDB code 1N4Y; shown in red), which is a potent platelet-aggregation inhibitor from snake venom [32] and cardosin A (PDB code 1B5F; shown in blue) are represented by their C-alpha backbones. The protruding RGD motif in kistrin is shown in white, and the KGE motif in cardosin A is shown in yellow.

RGD-mediated recognition events is therefore an interesting novel observation. C2 domains are found in a large number of eukaryotic proteins and are known to bind phospholipids in a calcium-dependent manner [30,49]. In proteins such as synaptotagmin and phospholipase A2, C2 domains have also been shown to mediate protein–protein interactions, and it was recently demonstrated that they may also work as phosphotyrosine-recognition domains [50–53]. The findings described here show that the C2 domain of PLDα may act as a protein-binding domain in addition to its role in Ca^{2+}-dependent phospholipid binding [54]. It remains to be established if this new role as an RGD-binding domain is exclusive to the PLDα C2 domain or is common to other C2-containing proteins. The identification of more plant proteins that interact with C2 domains will certainly give new insights into their involvement as signalling modules in plant systems.

Experimental procedures

Plant material

The parts of *C. cardunculus* L were collected in the field between June and July, and, except for the seeds which were stored at room temperature, all the other parts were frozen immediately in liquid nitrogen, and kept at −80 °C until use.

Purification of cardosin A-interacting protein

Pollen (200 mg) was ground in a mortar and pestle under liquid nitrogen, and the proteins were extracted in 1 mL Tris-buffered saline (NaCl/Tris, pH 7.0) containing 3 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A and 200 mM octyl glucoside. The extract was centrifuged at 12 000 ***g*** for 20 min (4 °C), and the supernatant (800 μL) was applied to a NHFRGDHTK–EAH Sepharose 4B column (1 mL bead volume). EAH Sepharose (Amersham Biosciences, Uppsala, Sweden) preparation and peptide ligation were performed according to the manufacturer's instructions. The column was pre-equilibrated with NaCl/Tris, pH 7.0, containing 3 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A and 50 mM octyl glucoside (column buffer) and incubated overnight at 4 °C with the extract. After the column had been washed with 5 mL column buffer, it was eluted with 5 mL column buffer containing RGDS peptide (1 $mg \cdot mL^{-1}$; Sigma). The purified proteins were analyzed by SDS/PAGE, and amino-acid sequence information was obtained by MS analysis.

MS analysis

For identification of proteins purified by NHFRGDHTK–EAH Sepharose 4B affinity column, bands were excised from Coomassie-stained SDS/polyacrylamide gels and in-gel digested with trypsin. The resulting peptide mixture was desalted using ZipTips (Millipore Corp., Billerica, MA, USA) and analyzed by nanoelectrospray MS. Mass spectra were acquired on a hybrid quadrupole time-of-flight mass spectrometer (Q-Tof; Micromass, Manchester, UK). The peptide sequence tag method [55] and *de novo* sequencing were used to identify the protein.

Extract preparation and immunoprecipitation

Mature pistils (200 mg) were ground in a mortar and pestle under liquid nitrogen, and proteins were extracted in NaCl/Tris containing 1% Triton X-100, 1 μM pepstatin A plus a protease inhibitor cocktail (Roche Diagnostics GmbH) (immunoprecipitation buffer). The extract was centrifuged for 20 min at 12 000 ***g*** (4 °C), and the supernatant (500 μL) was incubated overnight at 4 °C with 3 μg PLD polyclonal antibody (commercially purified antibody produced against PLD isolated from cabbage; Nordic Immunological Laboratories, Tilburg, the Netherlands). The samples were then incubated for 60 min at 4 °C with 100 μL protein A–Sepharose beads (Amersham Biosciences) and sequentially washed with immunoprecipitation buffer,

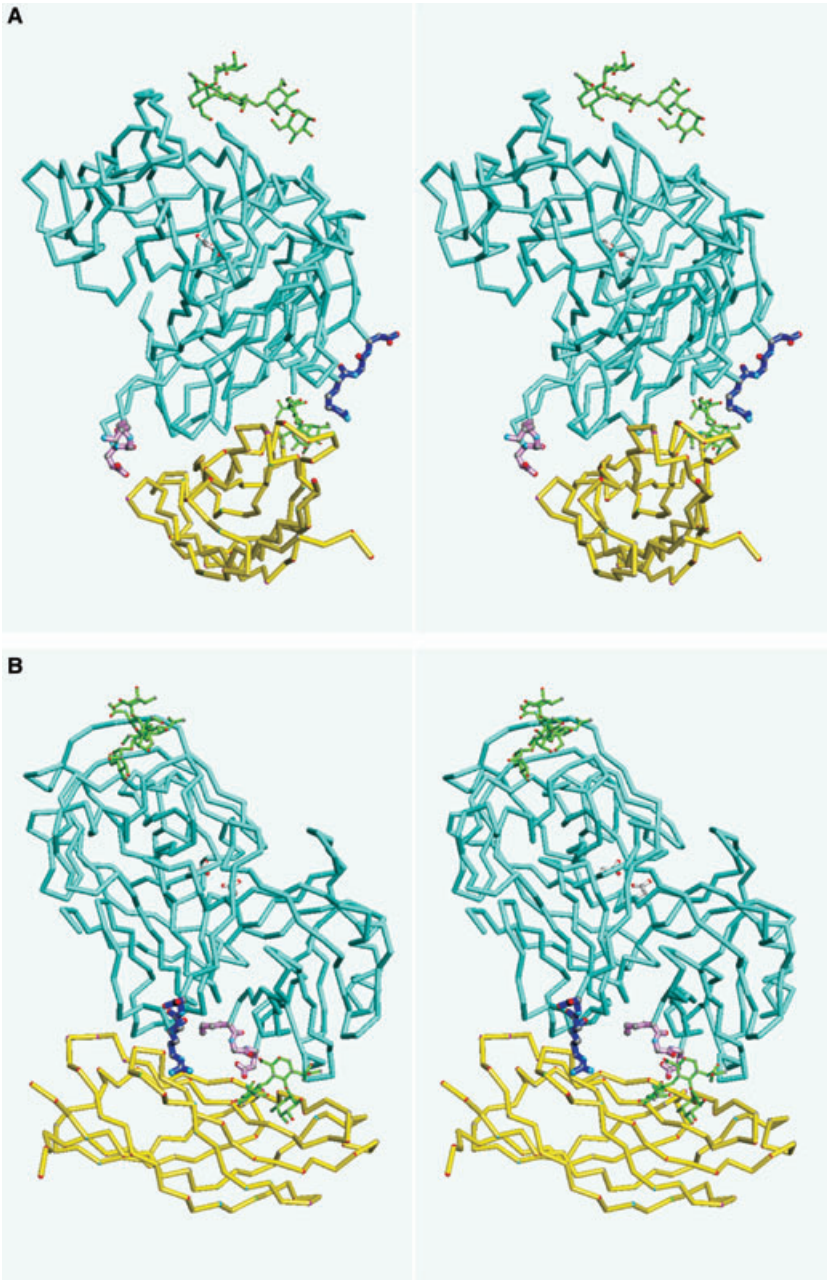

Fig. 9. Docking model of cardosin A and C2 domain. (A) The C-alpha backbone of cardosin A (PDB code 1BF5) is represented in cyan with sugars shown in green and the catalytic aspartates in white (centre of cyan protein structure). The RGD and KGE motifs are represented with all nonhydrogen atoms in blue and pink, respectively. The structure of the C2 domain of human PLA2 (PDB code 1RLW) was docked manually to the aspartic proteinase such that it established strong protein–protein interactions and contacted the RGD motif as well as the KGE sequence. (B) Same proteins as in (A) but picture rotated by ≈90 ° around the *y*-axis.

immunoprecipitation buffer containing 250 mM NaCl, and the same buffer without Triton X-100. The immunoprecipitated proteins were eluted from the beads by boiling in 2 × Laemmli sample buffer for subsequent analysis by SDS/PAGE and immunoblotting.

cDNA cloning of *C. cardunculus* L PLDα

Total RNA was isolated from pollen and immature pistils using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and poly(A)$^+$ mRNA was purified using the mRNA Purification Kit (Amersham Biosciences). Immature pistil mRNA was used in the construction of a λTriplEx cDNA library as follows. A TimeSaver cDNA Synthesis Kit (Amersham Biosciences) was used to generate a cDNA library with cohesive *Eco*RI sites, and cDNA was ligated to λTriplEx arms according to the supplier's protocol (Clontech, Palo Alto, CA, USA). The λTriplEx packaging reactions were performed as described in the Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA, USA) instruction manual, and the subsequent cDNA library amplification and titre calculation were performed according to the λTriplEx user manual (Clontech). Pollen mRNA was used to generate an adap-

tor-ligated double-stranded cDNA RACE library with the Marathon cDNA Amplification Kit (Clontech) and with the 5′/3′ RACE kit, 2nd Generation (Roche, Basel, Switzerland). These cDNA libraries were subjected to PCR with degenerate primers that were designed according to the partial amino-acid sequences obtained by MS/MS and Edman degradation or to highly conserved domains of known plant PLDαs. The primers used were 5′-GAY GAYAAYCCWATYGGNGCWAC-3′ (forward) for the amino-acid sequence DDNPIGAT, 5′-WGCRTTRATRT AWGCRTCYTGRAT-3′ (reverse) for the sequence IQDAYINA, 5′-GARCCWTGGCAYGAYATYCAYWS-3′ (forward) for EPWHDIHS and 5′-ATGATGATYGTKGA YGAYGARTA-3′ (forward) for the sequence MMIVD DEY. Based on the PCR-amplified cDNA fragments, a specific primer 5′-GAGAACCGACGCTTTATGATCTACG TGC (forward) coding for the sequence ENRRFMIYVH was synthesized to amplify the 3′ region of cardoon PLDα when used with a specific primer for the λTriplEx arms, 5′-TAATACGACTCACTATAGGG-3′ (reverse). The 5′ region of cardoon PLDα was amplified with the specific primer 5′-TAGCTTCACATGGATCTTAGAACC-3′ (reverse) coding for the sequence GSKIHVKL when used with the 5′ RACE anchor primer, 5′-GACCACGCGTATCGATGT CGAC-3′ (Roche). The PCR products were cloned, and both strands were sequenced by automated DNA sequencing.

GST fusion proteins cDNA coding for full-length PLDα were amplified by PCR using specific primers that include restriction sites for *Bam*HI and *Sal*I. The PCR-amplified product was subcloned in pGEX4T-2 vector (Amersham Biosciences). cDNA coding for the C2 domain of PLDα (construct coding amino acids 1–150) was amplified by PCR using *C. cardunculus* L and *A. thaliana* PLDα full-length cDNA as the template and inserted into *Bam*HI/*Sal*I sites of pGEX4T-2 vector (Amersham Biosciences). The positive clones selected by restriction analysis were confirmed by DNA sequencing. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) strain, and the recombinant proteins were expressed as fusion proteins with GST. The cells were grown at 28 °C until D_{600} of 0.8, and then the temperature was lowered to 20 °C. After an hour at this temperature, protein expression was induced by the addition of 0.1 mM isopropyl thio-β-D-galactoside, and the incubation continued for another 15 h. The fusion proteins were purified as described by Egas *et al.* [56]. Briefly, the cells were harvested by centrifugation at 8000 ***g*** for 10 min (4 °C) and washed with 10 mM Na_2HPO_4/1.8 mM KH_2PO_4/137 mM NaCl/2.7 mM KCl/1 mM $CaCl_2$/2 mM $MgCl_2$, pH 7.3 (NaCl/P_i). The cells were resuspended in 10 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 mM EDTA containing lysozyme (100 μg·mL^{-1}) and kept on ice for 15 min. Dithiothreitol was added to a final concentration of 5 mM. The proteins were then solubilized by the addition of *N*-laurylsarcosine to a final concentration of 0.25%, and the resulting mixture was frozen at −80 °C. After the proteins had been thawed, 2 mM $MgCl_2$ and 2 U·mL^{-1} DNase was added, and the solution was maintained for 2 h at 4 °C. The insoluble fraction was removed by centrifugation (15 000 ***g***, 15 min, 4 °C), and Triton X-100 was added to the supernatant at the same molar ratio as *N*-laurylsarcosine. The protein solutions were incubated for 30 min with the affinity resin glutathione–Sepharose (Amersham Biosciences), and the fusion proteins were purified according to the manufacturer's instructions. Recombinant proteins were dialysed overnight against NaCl/Tris. GST was produced by the above procedure using the vector pGEX4T-2 without insert.

Recombinant pCA and mutated pCA

pCA cDNA was cloned in the vector pET23a (Novagene, Madison, WI, USA) as described previously [7]. The QuikChange Site-Directed Mutagenesis kit (Stratagene) was used to generate pCA mutants in the vector pET23a. The following mutants were generated (mutations underlined): pCA(R246A) forward primer, 5′-CCTAATCATTTTGCG GGTGACCACACATATGTCCCTGTGAC-3′ (the reverse primer was the complementary sequence); pCA(D248A) forward primer, 5′-CCTAATCATTTTAGGGGTGCCCA CACATATGTCCCTGTGAC-3′ (the reverse primer was the complementary sequence); pCA(K455A) forward primer, 5′-CATCTTGAAAGTCGGTGCGGGAGAAGCAA CACAATGC-3′ (the reverse primer was the complementary sequence); pCA(E457A) forward primer, 5′-CATCTTGA AAGTCGGTAAGGGAGCAGCAACACAATGC-3′ (the reverse primer was the complementary sequence). The double mutant pCA(R246A/K455A) was generated sequentially using the specific primers described above. The positive mutant clones were confirmed by DNA sequencing. The constructs pCA wild-type and the mutants pCA(R246A), pCA(D248A), pCA(K455A), pCA(E457A) and pCA(R246A/K455A) were transformed into the *E. coli* BL21 (DE3) strain. The recombinant proteins were purified as described by Castanheira *et al.* [34]. After growth of the cells at 37 °C to D_{600} of 0.6, protein expression was induced by the addition of isopropyl thio-β-D-galactoside (0.5 mM final concentration). After 3 h, cells were harvested by centrifugation, resuspended in 50 mM Tris/50 mM NaCl (pH 7.4) and lysed with lysozyme (100 μg·mL^{-1}). After freezing and thawing, DNase (100 μg·mL^{-1}) and $MgCl_2$ (100 mM) were added, and the reaction mixture was incubated at 4 °C for 1 h. The cell lysate was then diluted into 1 L 50 mM Tris/50 mM NaCl (pH 7.4) and washed for 3 h at 4 °C with agitation. Then, the material was centrifuged at 10 000 ***g*** and washed again for another 3 h with 50 mM Tris/50 mM NaCl (pH 7.4) containing 0.1% (v/v) Triton X-100. After centrifugation at 10 000 ***g***, the purified inclusion bodies were dissolved in 8 M urea, with 100 mM 2-mercaptoethanol and then diluted (20-fold) with 20 mM

Tris/HCl, pH 8.0. The protein was then concentrated in a tangential flow ultrafiltration system (Pellicon 2; Millipore) and applied to an S-300 gel filtration column equilibrated in 20 mM Tris/0.4 M urea, pH 8.0 buffer. The protein fractions were further purified by ion-exchange chromatography with a Resource Q (Amersham Biosciences) column in an FPLC system using the buffer used for S-300 chromatography. Elution was carried out with a linear gradient of NaCl (0–0.5 M) at a flow rate of 1.0 $mL \cdot min^{-1}$. The wild-type and mutated forms of recombinant cardosin A were autoactivated and assayed for activity as described by Castanheira *et al*. [34].

Binding assays

In vitro interactions between native cardosin B, native cardosin A, recombinant wild-type pCA or pCA mutants and PLDα or C2 GST fusion proteins were examined by pull-down assays. Each GST fusion protein (10 μg) was incubated overnight with 10 μg native cardosins or recombinant cardosin A (wild-type and mutants), at 4 °C. When applicable, pepstatin A was used at a final concentration of 1 μM, and a 100-fold excess of NHFRGDHT peptide was used in the binding assays. The protein mixture was then incubated for 30 min with 40 μL glutathione–Sepharose beads (4 °C). The beads were extensively washed with NaCl/Tris containing 1% Triton X-100 and 500 mM NaCl. Beads were eluted in 2 × Laemmli sample buffer, and eluates were subjected to SDS/PAGE and immunoblotting. Native cardosin A and cardosin B used in the binding assays were purified from mature pistils of *C. cardunculus* L as described previously [6].

Gel electrophoresis and immunoblotting

Protein samples were separated by SDS/PAGE (12% acrylamide gels), and transferred to poly(vinylidene difluoride) membrane for immunoblotting (40 V, overnight, at 10 °C). The membranes were blocked for 60 min with 5% (w/v) nonfat dry milk plus 0.1% (v/v) Tween 20 in NaCl/Tris and then incubated at room temperature for 60 min with primary antibodies against PLD (Nordic Immunological Laboratories; 1 : 20 000 dilution), recombinant cardosin A (1 : 500), recombinant cardosin B (1 : 200) or GST (1 : 2000). After several washes with 0.5% (w/v) nonfat dry milk plus 0.1% (v/v) Tween 20 in NaCl/Tris, the membranes were incubated at room temperature for 60 min with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody against PLD (1 : 20 000), alkaline phosphatase-conjugated rabbit anti-goat secondary antibody against GST (1 : 10 000) or horseradish peroxidase-conjugated swine anti-rabbit antibody against recombinant cardosin A, recombinant cardosin B or PLD (1 : 1000) staining. The membranes were again washed, and immunostaining was visualized in two different ways. Peroxidase activity was developed by luminol chemiluminescence using the ECL method (Amersham Biosciences). Alkaline phosphatase activity was visualized by the enhanced chemifluorescence method on a Storm 860 gel and blot imaging system (Amersham Biosciences).

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